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(54) Title: NOVEL PROSTATE-ASSOCIATED KALLIKREIN
(57) Abstract
<p>The present invention provides a human prostate-associated kallikrein (HPAK) and polynucleotides which identify and encode HPAK. The invention also provides genetically engineered expression vectors and host cells comprising the nucleic acid sequences encoding HPAK and a method for producing HPAK. The invention also provides for antibodies or antagonists specifically binding HPAK, and their use, in the prevention and treatment of diseases associated with expression of HPAK. Additionally, the invention provides for the use of antisense molecules to polynucleotides encoding HPAK for the treatment of diseases associated with the expression of HPAK. The invention also provides diagnostic assays which utilize the polynucleotide, or fragments or the complement thereof, and antibodies specifically binding HPAK.</p>

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## NOVEL PROSTATE-ASSOCIATED KALLIKREIN TECHNICAL FIELD

This invention relates to nucleic acid and amino acid sequences of a novel prostate-associated kallikrein and to the use of these sequences in the diagnosis, prevention, and treatment 5 of cancer and disorders of the prostate.

### BACKGROUND ART

Prostate-specific antigen (PSA) is a 33 kD glycoprotein synthesized in the epithelial cells of the prostate gland. It is an secreted serine protease of the kallikrein family. PSA has been shown to digest the seminal vesicle protein, semenogelin, parathyroid hormone-related protein, 10 and insulin-like growth factor-binding protein-3 (Henttu P. et al. (1994) Ann. Med. 26: 157-164; Cramer S. D. et al. (1996) J. Urol. 156: 526-531).

Genes encoding the three human kallikreins, tissue kallikrein (KLK1), glandular kallikrein (KLK2), and PSA are located in a cluster at chromosome map position 19q13.2-q13.4 (Riegmen P.H. (1992) Genomics 14: 6-11). PSA shares more extensive homology with KLK2 15 than with KLK1. Both PSA and KLK2 are produced by prostate epithelial cells and their expression is regulated by androgens. Three amino acid residues were found to be critical for serine protease activity, residues H<sub>65</sub>, D<sub>120</sub>, and S<sub>213</sub> in PSA (Bridon D. P. et al. (1995) Urology 45: 801-806). Substrate specificity, described as chymotrypsinogen-like (with KLK2) or trypsin-like (with PSA) is thought to be determined by S<sub>207</sub> in PSA and D<sub>209</sub> in KLK2 (Bridon et al., 20 supra). KLK1 is chymotrypsinogen-like and expressed in the pancreas, urinary system, and sublingual gland. KLK1, like the other kallikreins, is made as a pre-pro-protein and is processed into an active form of 238 amino acids by cleavage of a 24 amino acid terminal signal sequence (Fukushima D. et al. (1985) Biochemistry 24: 8037-8043).

Adenocarcinoma of the prostate accounts for a significant number of malignancies in men 25 over 50, with over 122,000 new cases occurring per year, in the United States alone. Prostate-specific antigen (PSA) is the most sensitive marker available for monitoring cancer progression and response to therapy. Serum PSA is elevated in up to 92% of patients with prostatic carcinoma, depending upon tumor volume. Since PSA is also moderately elevated in patients with benign prostate hyperplasia, additional techniques are needed to distinguish between the 30 two.

Discovery of proteins related to PSA and the polynucleotides encoding them satisfies a need in the art by providing new compositions useful in diagnosis, prevention, and treatment of

cancer and disorders of the prostate.

## DISCLOSURE OF THE INVENTION

The present invention features a novel prostate-associated kallikrein hereinafter designated HPAK and characterized as having chemical and structural similarity to human 5 pancreatic kallikrein and other kallikreins.

Accordingly, the invention features a substantially purified HPAK which has the amino acid sequence shown in SEQ ID NO:1.

One aspect of the invention features isolated and substantially purified polynucleotides that encode HPAK. In a particular aspect, the polynucleotide is the nucleotide sequence of SEQ 10 ID NO:2.

The invention also relates to a polynucleotide sequence comprising the complement of SEQ ID NO:2 or variants thereof. In addition, the invention features polynucleotide sequences which hybridize under stringent conditions to SEQ ID NO:2.

The invention additionally features nucleic acid sequences encoding polypeptides. 15 oligonucleotides, peptide nucleic acids (PNA), fragments, portions or antisense molecules thereof, and expression vectors and host cells comprising polynucleotides that encode HPAK. The present invention also features antibodies which bind specifically to HPAK, and pharmaceutical compositions comprising substantially purified HPAK. The invention also features the use of agonists and antagonists of HPAK. The invention also features a method for 20 producing HPAK using the host cell, and methods for treating cancer and prostatic hyperplasia by administering an antagonist to HPAK.

## BRIEF DESCRIPTION OF DRAWINGS

Figures 1A, 1B and 1C show the amino acid sequence (SEQ ID NO:1) and nucleic acid sequence (SEQ ID NO:2) of HPAK. The alignment was produced using MacDNASIS PRO™ 25 software (Hitachi Software Engineering Co., Ltd., San Bruno, CA).

Figures 2A and 2B shows the amino acid sequence alignments among HPAK (SEQ ID NO:1), human pancreatic kallikrein (GI 186653; SEQ ID NO:3), and African rat renal kallikrein (GI 55527; SEQ ID NO:4). The alignment was produced using the multisequence alignment program of DNASTAR™ software (DNASTAR Inc. Madison WI).

30 Figure 3 shows the hydrophobicity plot (MacDNASIS PRO software) for HPAK. SEQ ID NO: 1: the positive X axis reflects amino acid position, and the negative Y axis, hydrophobicity.

Figure 4 shows the hydrophobicity plot for human pancreatic kallikrein. SEQ ID NO:3.

Figure 5 shows the northern analysis for SEQ ID NO:2. The northern analysis was produced electronically using LIFESEQ™ database (Incyte Pharmaceuticals, Inc., Palo Alto, CA).

### **MODES FOR CARRYING OUT THE INVENTION**

5 Before the present proteins, nucleotide sequences, and methods are described, it is understood that this invention is not limited to the particular methodology, protocols, cell lines, vectors, and reagents described as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended 10 claims.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a host cell" includes a plurality of such host cells, reference to the "antibody" is a reference to one or more antibodies and equivalents thereof known to those 15 skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and 20 materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies which are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

### **25 DEFINITIONS**

"Nucleic acid sequence" as used herein refers to an oligonucleotide, nucleotide, or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand. Similarly, "amino acid sequence" as used herein refers to an oligopeptide, peptide, polypeptide, 30 or protein sequence, and fragments or portions thereof, and to naturally occurring or synthetic molecules.

Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a

naturally occurring protein molecule. "amino acid sequence" and like terms, such as "polypeptide" or "protein" are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

"Peptide nucleic acid", as used herein, refers to a molecule which comprises an oligomer 5 to which an amino acid residue, such as lysine, and an amino group have been added. These small molecules, also designated anti-gene agents, stop transcript elongation by binding to their complementary strand of nucleic acid (Nielsen, P.E. et al. (1993) *Anticancer Drug Des.* 8:53-63).

HPAK, as used herein, refers to the amino acid sequences of substantially purified HPAK obtained from any species, particularly mammalian, including bovine, ovine, porcine, murine, 10 equine, and preferably human, from any source whether natural, synthetic, semi-synthetic, or recombinant.

"Consensus", as used herein, refers to a nucleic acid sequence which has been resequenced to resolve uncalled bases, or which has been extended using XL-PCR™ (Perkin Elmer, Norwalk, CT) in the 5' and/or the 3' direction and resequenced, or which has been 15 assembled from the overlapping sequences of more than one Incyte clone using the GELVIEW™ Fragment Assembly system (GCG, Madison, WI), or which has been both extended and assembled.

A "variant" of HPAK, as used herein, refers to an amino acid sequence that is altered by one or more amino acids. The variant may have "conservative" changes, wherein a substituted 20 amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Similar minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity may be found using 25 computer programs well known in the art, for example, DNASTAR software.

A "deletion", as used herein, refers to a change in either amino acid or nucleotide sequence in which one or more amino acid or nucleotide residues, respectively, are absent.

An "insertion" or "addition", as used herein, refers to a change in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid or nucleotide residues, 30 respectively, as compared to the naturally occurring molecule.

A "substitution", as used herein, refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

The term "biologically active", as used herein, refers to a protein having structural, regulatory, or biochemical functions of a naturally occurring molecule. Likewise, "immunologically active" refers to the capability of the natural, recombinant, or synthetic HPAK, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells 5 and to bind with specific antibodies.

The term "agonist", as used herein, refers to a molecule which, when bound to HPAK, causes a change in HPAK which modulates the activity of HPAK. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules which bind to HPAK.

The terms "antagonist" or "inhibitor", as used herein, refer to a molecule which, when 10 bound to HPAK, blocks or modulates the biological or immunological activity of HPAK.

Antagonists and inhibitors may include proteins, nucleic acids, carbohydrates, or any other molecules which bind to HPAK.

The term "modulate", as used herein, refers to a change or an alteration in the biological activity of HPAK. Modulation may be an increase or a decrease in protein activity, a change in 15 binding characteristics, or any other change in the biological, functional or immunological properties of HPAK.

The term "mimetic", as used herein, refers to a molecule, the structure of which is developed from knowledge of the structure of HPAK or portions thereof and, as such, is able to effect some or all of the actions of kallikrein-like molecules.

20 The term "derivative", as used herein, refers to the chemical modification of a nucleic acid encoding HPAK or the encoded HPAK. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group. A nucleic acid derivative would encode a polypeptide which retains essential biological characteristics of the natural molecule.

The term "substantially purified", as used herein, refers to nucleic or amino acid 25 sequences that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated.

"Amplification" as used herein refers to the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction (PCR) technologies 30 well known in the art (Dieffenbach, C.W. and G.S. Dveksler (1995) PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview, NY).

The term "hybridization", as used herein, refers to any process by which a strand of

nucleic acid binds with a complementary strand through base pairing.

The term "hybridization complex", as used herein, refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary G and C bases and between complementary A and T bases; these hydrogen bonds may be further 5 stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution (e.g., C.t or R.t and -sis) or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (e.g., membranes, filters, chips, pins or glass slides to which cells have been fixed for *in situ* hybridization).

10 The terms "complementary" or "complementarity", as used herein, refer to the natural binding of polynucleotides under permissive salt and temperature conditions by base-pairing. For example, for the sequence "A-G-T" binds to the complementary sequence "T-C-A". Complementarity between two single-stranded molecules may be "partial", in which only some of the nucleic acids bind, or it may be complete when total complementarity exists between the 15 single stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, which depend upon binding between nucleic acids strands.

The term "homology", as used herein, refers to a degree of complementarity. There may 20 be partial homology or complete homology (i.e., identity). A partially complementary sequence is one that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid; it is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or northern blot, solution hybridization and the 25 like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (i.e., the hybridization) of a completely homologous sequence or probe to the target sequence under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) 30 interaction. The absence of non-specific binding may be tested by the use of a second target sequence which lacks even a partial degree of complementarity (e.g., less than about 30% identity); in the absence of non-specific binding, the probe will not hybridize to the second non-

complementary target sequence.

As known in the art, numerous equivalent conditions may be employed to comprise either low or high stringency conditions. Factors such as the length and nature (DNA, RNA, base composition) of the sequence, nature of the target (DNA, RNA, base composition, presence in solution or immobilization, etc.), and the concentration of the salts and other components (e.g., the presence or absence of formamide, dextran sulfate and/or polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency different from, but equivalent to, the above listed conditions.

The term "stringent conditions", as used herein, is the "stringency" which occurs within a range from about  $T_m - 5^\circ\text{C}$  ( $5^\circ\text{C}$  below the melting temperature ( $T_m$ ) of the probe) to about  $20^\circ\text{C}$  to  $25^\circ\text{C}$  below  $T_m$ . As will be understood by those of skill in the art, the stringency of hybridization may be altered in order to identify or detect identical or related polynucleotide sequences.

The term "antisense", as used herein, refers to nucleotide sequences which are complementary to a specific DNA or RNA sequence. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. Antisense molecules may be produced by any method, including synthesis by ligating the gene(s) of interest in a reverse orientation to a viral promoter which permits the synthesis of a complementary strand. Once introduced into a cell, this transcribed strand combines with natural sequences produced by the cell to form duplexes. These duplexes then block either the further transcription or translation. In this manner, mutant phenotypes may be generated. The designation "negative" is sometimes used in reference to the antisense strand, and "positive" is sometimes used in reference to the sense strand.

The term "portion", as used herein, with regard to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid. Thus, a protein "comprising at least a portion of the amino acid sequence of SEQ ID NO:1" encompasses the full-length human HPAK and fragments thereof.

"Transformation", as defined herein, describes a process by which exogenous DNA enters and changes a recipient cell. It may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is

selected based on the host cell being transformed and may include, but is not limited to, viral infection, electroporation, lipofection, and particle bombardment. Such "transformed" cells include stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome. They also include cells 5 which transiently express the inserted DNA or RNA for limited periods of time.

The term "antigenic determinant", as used herein, refers to that portion of a molecule that makes contact with a particular antibody (i.e., an epitope). When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure 10 on the protein; these regions or structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (i.e., the immunogen used to elicit the immune response) for binding to an antibody.

The terms "specific binding" or "specifically binding", as used herein, in reference to the interaction of an antibody and a protein or peptide, mean that the interaction is dependent upon 15 the presence of a particular structure (i.e., the antigenic determinant or epitope) on the protein; in other words, the antibody is recognizing and binding to a specific protein structure rather than to proteins in general. For example, if an antibody is specific for epitope "A", the presence of a protein containing epitope A (or free, unlabeled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody.

20 The term "sample", as used herein, is used in its broadest sense. A biological sample suspected of containing nucleic acid encoding HPAK or fragments thereof may comprise a cell, chromosomes isolated from a cell (e.g., a spread of metaphase chromosomes), genomic DNA (in solution or bound to a solid support such as for Southern analysis), RNA (in solution or bound to a solid support such as for northern analysis), cDNA (in solution or bound to a solid support), an 25 extract from cells or a tissue, and the like.

The term "correlates with expression of a polynucleotide", as used herein, indicates that the detection of the presence of ribonucleic acid that is similar to SEQ ID NO:2 by northern analysis is indicative of the presence of mRNA encoding HPAK in a sample and thereby correlates with expression of the transcript from the polynucleotide encoding the protein.

30 "Alterations" in the polynucleotide of SEQ ID NO: 2, as used herein, comprise any alteration in the sequence of polynucleotides encoding HPAK including deletions, insertions, and point mutations that may be detected using hybridization assays. Included within this definition

is the detection of alterations to the genomic DNA sequence which encodes HPAK (e.g., by alterations in the pattern of restriction fragment length polymorphisms capable of hybridizing to SEQ ID NO:2), the inability of a selected fragment of SEQ ID NO. 2 to hybridize to a sample of genomic DNA (e.g., using allele-specific oligonucleotide probes), and improper or unexpected 5 hybridization, such as hybridization to a locus other than the normal chromosomal locus for the polynucleotide sequence encoding HPAK (e.g., using fluorescent *in situ* hybridization [FISH] to metaphase chromosomes spreads).

As used herein, the term "antibody" refers to intact molecules as well as fragments thereof, such as Fa, F(ab'), and Fv, which are capable of binding the epitopic determinant.

10 Antibodies that bind HPAK polypeptides can be prepared using intact polypeptides or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or peptide used to immunize an animal can be derived from the transition of RNA or synthesized chemically, and can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin and thyroglobulin. The coupled peptide is 15 then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

The term "humanized antibody", as used herein, refers to antibody molecules in which amino acids have been replaced in the non-antigen binding regions in order to more closely resemble a human antibody, while still retaining the original binding ability.

#### THE INVENTION

20 The invention is based on the discovery of a novel human kallikrein, (HPAK), the polynucleotides encoding HPAK, and the use of these compositions for the diagnosis, prevention, or treatment of cancer and disorders of the prostate.

Nucleic acids encoding the human HPAK of the present invention were first identified in 25 Incyte Clone 964204 from the breast tissue cDNA library (BRSTNOT05) through a computer-generated search for amino acid sequence alignments. A consensus sequence, SEQ ID NO:2, was derived from the following overlapping and/or extended nucleic acid sequences: Incyte Clones 964204 (BRSTNOT05), 875949 (LUNGAST01), 1325870 (LPARNOT02), 1685649 (PROSTNOT15), and 1725220 (PROSNOT14).

In one embodiment, the invention encompasses a polypeptide comprising the amino acid 30 sequence of SEQ ID NO:1, as shown in Figs. 1A and 1B. HPAK is 253 amino acids in length and has four potential N-glycosylation sites at asparagine residues 102, 168, 184, and 213. HPAK has chemical and structural homology with human pancreatic kallikrein (GI 186653; SEQ

ID NO:3). In particular, HPAK and human pancreatic kallikrein share 54% identity. HPAK's amino terminal 24 amino acids are hydrophilic and closely resemble signal sequences important for kallikrein secretion (Figures 2A, 2B, 3, and 4). HPAK sequence contains conserved residues critical for serine protease activity, H<sub>65</sub>, D<sub>113</sub>, and S<sub>206</sub> (Figures 2A and 2B). Amino acid residue 5 D<sub>200</sub> is likely to confer on HPAK chymotrypsinogen-like activity. HPAK amino acid sequence includes 10 conserved cysteine residues (31, 50, 66, 145, 166, 177, 191, 202, 212, and 227; Figures 2A and 2B). In the kallikreins mentioned above, these cysteines are structurally important and form five disulfide bonds. As illustrated by Figures 3 and 4, HPAK and human pancreatic kallikrein have rather similar hydrophobicity plots. Northern analysis reveals the 10 expression pattern of this sequence in various libraries (Figure 5). Of the 15 tissues in which HPAK is expressed, six are from the prostate gland and seven come from cancer patients.

The invention also encompasses HPAK variants. A preferred HPAK variant is one having at least 80%, and more preferably 90%, amino acid sequence similarity to the HPAK amino acid sequence (SEQ ID NO:1). A most preferred HPAK variant is one having at least 15 95% amino acid sequence similarity to SEQ ID NO:1.

The invention also encompasses polynucleotides which encode HPAK. Accordingly, any nucleic acid sequence which encodes the amino acid sequence of HPAK can be used to generate recombinant molecules which express HPAK. In a particular embodiment, the invention encompasses the polynucleotide comprising the nucleic acid sequence of SEQ ID NO:2 20 as shown in Figures 1A, 1B and 1C.

It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding HPAK, some bearing minimal homology to the nucleotide sequences of any known and naturally occurring gene, may be produced. Thus, the invention contemplates each and every possible variation of nucleotide 25 sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring HPAK, and all such variations are to be considered as being specifically disclosed.

Although nucleotide sequences which encode HPAK and its variants are preferably 30 capable of hybridizing to the nucleotide sequence of the naturally occurring HPAK under appropriately selected conditions of stringency, it may be advantageous to produce nucleotide sequences encoding HPAK or its derivatives possessing a substantially different codon usage.

Codons may be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding HPAK and its derivatives without altering the encoded amino acid sequences include

5 the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

The invention also encompasses production of DNA sequences, or portions thereof, which encode HPAK and its derivatives, entirely by synthetic chemistry. After production, the synthetic sequence may be inserted into any of the many available expression vectors and cell

10 systems using reagents that are well known in the art at the time of the filing of this application. Moreover, synthetic chemistry may be used to introduce mutations into a sequence encoding HPAK or any portion thereof.

Also encompassed by the invention are polynucleotide sequences that are capable of hybridizing to the claimed nucleotide sequences, and in particular, those shown in SEQ ID NO:2, 15 under various conditions of stringency. Hybridization conditions are based on the melting temperature (T<sub>m</sub>) of the nucleic acid binding complex or probe, as taught in Wahl, G.M. and S.L. Berger (1987: Methods Enzymol. 152:399-407) and Kimmel, A.R. (1987: Methods Enzymol. 152:507-511), and may be used at a defined stringency.

Altered nucleic acid sequences encoding HPAK which are encompassed by the invention 20 include deletions, insertions, or substitutions of different nucleotides resulting in a polynucleotide that encodes the same or a functionally equivalent HPAK. The encoded protein may also contain deletions, insertions, or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent HPAK. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the 25 amphipathic nature of the residues as long as the biological activity of HPAK is retained. For example, negatively charged amino acids may include aspartic acid and glutamic acid; positively charged amino acids may include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values may include leucine, isoleucine, and valine; glycine and alanine; asparagine and glutamine; serine and threonine; phenylalanine and tyrosine.

30 Also included within the scope of the present invention are alleles of the genes encoding HPAK. As used herein, an "allele" or "allelic sequence" is an alternative form of the gene which may result from at least one mutation in the nucleic acid sequence. Alleles may result in altered

mRNAs or polypeptides whose structure or function may or may not be altered. Any given gene may have none, one, or many allelic forms. Common mutational changes which give rise to alleles are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times 5 in a given sequence.

Methods for DNA sequencing which are well known and generally available in the art may be used to practice any embodiments of the invention. The methods may employ such enzymes as the Klenow fragment of DNA polymerase I, Sequenase® (US Biochemical Corp., Cleveland, OH), Taq polymerase (Perkin Elmer), thermostable T7 polymerase (Amersham, 10 Chicago, IL), or combinations of recombinant polymerases and proofreading exonucleases such as the ELONGASE Amplification System marketed by Gibco BRL (Gaithersburg, MD). Preferably, the process is automated with machines such as the Hamilton Micro Lab 2200 (Hamilton, Reno, NV), Peltier Thermal Cycler (PTC200; MJ Research, Watertown, MA) and the ABI 377 DNA sequencers (Perkin Elmer).

15 The nucleic acid sequences encoding HPAK may be extended utilizing a partial nucleotide sequence and employing various methods known in the art to detect upstream sequences such as promoters and regulatory elements. For example, one method which may be employed, "restriction-site" PCR, uses universal primers to retrieve unknown sequence adjacent to a known locus (Sarkar, G. (1993) PCR Methods Applic. 2:318-322). In particular, genomic 20 DNA is first amplified in the presence of primer to linker sequence and a primer specific to the known region. The amplified sequences are then subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

25 Inverse PCR may also be used to amplify or extend sequences using divergent primers based on a known region (Triglia, T. et al. (1988) Nucleic Acids Res. 16:8186). The primers may be designed using OLIGO 4.06 Primer Analysis software (National Biosciences Inc., Plymouth, MN), or another appropriate program, to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68°-72° C. The method 30 uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template.

Another method which may be used is capture PCR which involves PCR amplification of

DNA fragments adjacent to a known sequence in human and yeast artificial chromosome DNA (Lagerstrom, M. et al. (1991) PCR Methods Applic. 1:111-119). In this method, multiple restriction enzyme digestions and ligations may also be used to place an engineered double-stranded sequence into an unknown portion of the DNA molecule before performing

5 PCR.

Another method which may be used to retrieve unknown sequences is that of Parker, J.D. et al. (1991; Nucleic Acids Res. 19:3055-3060). Additionally, one may use PCR, nested primers, and PromoterFinder™ libraries to walk in genomic DNA (Clontech, Palo Alto, CA). This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

10 When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected to include larger cDNAs. Also, random-primed libraries are preferable, in that they will contain more sequences which contain the 5' regions of genes. Use of a randomly primed library may be especially preferable for situations in which an oligo d(T) library does not yield a full-length cDNA. Genomic libraries may be useful for extension of sequence into the 5' and 3'

15 non-transcribed regulatory regions.

Capillary electrophoresis systems which are commercially available may be used to analyze the size or confirm the nucleotide sequence of sequencing or PCR products. In particular, capillary sequencing may employ flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection

20 of the emitted wavelengths by a charge coupled device camera. Output/light intensity may be converted to electrical signal using appropriate software (e.g. Genotyper™ and Sequence Navigator™, Perkin Elmer) and the entire process from loading of samples to computer analysis and electronic data display may be computer controlled. Capillary electrophoresis is especially preferable for the sequencing of small pieces of DNA which might be present in limited amounts

25 in a particular sample.

In another embodiment of the invention, polynucleotide sequences or fragments thereof which encode HPAK, or fusion proteins or functional equivalents thereof, may be used in recombinant DNA molecules to direct expression of HPAK in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the

30 same or a functionally equivalent amino acid sequence may be produced and these sequences may be used to clone and express HPAK.

As will be understood by those of skill in the art, it may be advantageous to produce

HPAK-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce a recombinant RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

The nucleotide sequences of the present invention can be engineered using methods generally known in the art in order to alter HPAK encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the gene product. DNA shuffling by random fragmentation and PCR reassembly of gene 10 fragments and synthetic oligonucleotides may be used to engineer the nucleotide sequences. For example, site-directed mutagenesis may be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, or introduce mutations, and so forth.

In another embodiment of the invention, natural, modified, or recombinant nucleic acid sequences encoding HPAK may be ligated to a heterologous sequence to encode a fusion protein. 15 For example, to screen peptide libraries for inhibitors of HPAK activity, it may be useful to encode a chimeric HPAK protein that can be recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the HPAK encoding sequence and the heterologous protein sequence, so that HPAK may be cleaved and purified away from the heterologous moiety.

20 In another embodiment, sequences encoding HPAK may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers, M.H. et al. (1980) *Nucl. Acids Res. Symp. Ser.* 215-223; Horn, T. et al. (1980) *Nucl. Acids Res. Symp. Ser.* 225-232). Alternatively, the protein itself may be produced using chemical methods to synthesize the amino acid sequence of HPAK, or a portion thereof. For example, peptide synthesis can be performed 25 using various solid-phase techniques (Roberge, J.Y. et al. (1995) *Science* 269:202-204) and automated synthesis may be achieved, for example, using the ABI 431A Peptide Synthesizer (Perkin Elmer).

The newly synthesized peptide may be substantially purified by preparative high performance liquid chromatography (e.g., Creighton, T. (1983) Proteins, Structures and 30 Molecular Principles, WH Freeman and Co., New York, NY). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton, supra). Additionally, the amino acid sequence of HPAK, or any part

thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide.

In order to express a biologically active HPAK, the nucleotide sequences encoding HPAK or functional equivalents, may be inserted into appropriate expression vector, i.e., a vector which 5 contains the necessary elements for the transcription and translation of the inserted coding sequence.

Methods which are well known to those skilled in the art may be used to construct expression vectors containing sequences encoding HPAK and appropriate transcriptional and translational control elements. These methods include in vitro recombinant DNA techniques, 10 synthetic techniques, and in vivo genetic recombination. Such techniques are described in Sambrook, J. et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, NY, and Ausubel, F.M. et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY.

A variety of expression vector/host systems may be utilized to contain and express 15 sequences encoding HPAK. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g., baculovirus); plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial 20 expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems.

The "control elements" or "regulatory sequences" are those non-translated regions of the vector--enhancers, promoters, 5' and 3' untranslated regions--which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable 25 transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the Bluescript® phagemid (Stratagene, LaJolla, CA) or pSport1™ plasmid (Gibco BRL) and the like may be used. The baculovirus polyhedrin promoter may be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock, 30 RUBISCO; and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) may be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell

line that contains multiple copies of the sequence encoding HPAK. vectors based on SV40 or EBV may be used with an appropriate selectable marker.

In bacterial systems, a number of expression vectors may be selected depending upon the use intended for HPAK. For example, when large quantities of HPAK are needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be used. Such vectors include, but are not limited to, the multifunctional *E. coli* cloning and expression vectors such as Bluescript® (Stratagene), in which the sequence encoding HPAK may be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of β-galactosidase so that a hybrid protein is produced; pIN vectors (Van Heeke, G. and S.M. Schuster (1989) *J. Biol. Chem.* 264:5503-5509); and the like. pGEX vectors (Promega, Madison, WI) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems may be designed to include heparin, thrombin, or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

In the yeast, *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH may be used. For reviews, see Ausubel et al. (supra) and Grant et al. (1987) *Methods Enzymol.* 153:516-544.

20 In cases where plant expression vectors are used, the expression of sequences encoding HPAK may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV (Takamatsu, N. (1987) *EMBO J.* 6:307-311). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used (Coruzzi, 25 G. et al. (1984) *EMBO J.* 3:1671-1680; Broglie, R. et al. (1984) *Science* 224:838-843; and Winter, J. et al. (1991) *Results Probl. Cell Differ.* 17:85-105). These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. Such techniques are described in a number of generally available reviews (see, for example, Hobbs, S. or Murry, L.E. in McGraw Hill *Yearbook of Science and Technology* (1992) McGraw 30 Hill, New York, NY; pp. 191-196.

An insect system may also be used to express HPAK. For example, in one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign

genes in Spodoptera frugiperda cells or in Trichoplusia larvae. The sequences encoding HPAK may be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of HPAK will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant 5 viruses may then be used to infect, for example, S. frugiperda cells or Trichoplusia larvae in which HPAK may be expressed (Engelhard, E.K. et al. (1994) Proc. Nat. Acad. Sci. 91:3224-3227)

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, sequences encoding HPAK may be 10 ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing HPAK in infected host cells (Logan, J. and Shenk, T. (1984) Proc. Natl. Acad. Sci. 81:3655-3659). In addition, transcription 15 enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding HPAK. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding HPAK, its initiation codon, and upstream 20 sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation 25 codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. et al. (1994) Results Probl. Cell Differ. 20:125-162).

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such 30 modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding

and/or function. Different host cells such as CHO, HeLa, MDCK, HEK293, and WI38, which have specific cellular machinery and characteristic mechanisms for such post-translational activities, may be chosen to ensure the correct modification and processing of the foreign protein.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express HPAK may be transformed using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance 10 to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M. et al. 15 (1977) Cell 11:223-32) and adenine phosphoribosyltransferase (Lowy, I. et al. (1980) Cell 22:817-23) genes which can be employed in tk<sup>-</sup> or aprt<sup>-</sup> cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. 77:3567-70); npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin, F. 20 et al (1981) J. Mol. Biol. 150:1-14) and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, supra). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine (Hartman, S.C. and R.C. Mulligan 25 (1988) Proc. Natl. Acad. Sci. 85:8047-51). Recently, the use of visible markers has gained popularity with such markers as anthocyanins,  $\beta$  glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes, C.A. et al. (1995) Methods Mol. Biol. 55:121-131). .

Although the presence/absence of marker gene expression suggests that the gene of 30 interest is also present, its presence and expression may need to be confirmed. For example, if the sequence encoding HPAK is inserted within a marker gene sequence, recombinant cells containing sequences encoding HPAK can be identified by the absence of marker gene function.

Alternatively, a marker gene can be placed in tandem with a sequence encoding HPAK under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem gene as well.

Alternatively, host cells which contain the nucleic acid sequence encoding HPAK and express HPAK may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include membrane, solution, or chip based technologies for the detection and/or quantification of nucleic acid or protein.

The presence of polynucleotide sequences encoding HPAK can be detected by

10 DNA-DNA or DNA-RNA hybridization or amplification using probes or portions or fragments of polynucleotides encoding HPAK. Nucleic acid amplification based assays involve the use of oligonucleotides or oligomers based on the sequences encoding HPAK to detect transformants containing DNA or RNA encoding HPAK. As used herein "oligonucleotides" or "oligomers" refer to a nucleic acid sequence of at least about 10 nucleotides and as many as about 60

15 nucleotides, preferably about 15 to 30 nucleotides, and more preferably about 20-25 nucleotides, which can be used as a probe or amplimer.

A variety of protocols for detecting and measuring the expression of HPAK, using either polyclonal or monoclonal antibodies specific for the protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and

20 fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on HPAK is preferred, but a competitive binding assay may be employed. These and other assays are described, among other places, in Hampton, R. et al. (1990: Serological Methods, a Laboratory Manual, APS Press, St Paul, MN) and Maddox, D.E. et al. (1983: *J. Exp. Med.* 158:1211-1216).

25 A wide variety of labels and conjugation techniques are known by those skilled in the art and may be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding HPAK include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the sequences encoding HPAK, or any portions thereof may be cloned

30 into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3, or SP6 and labeled nucleotides. These procedures

may be conducted using a variety of commercially available kits (Pharmacia & Upjohn, (Kalamazoo, MI); Promega (Madison WI); and U.S. Biochemical Corp., Cleveland, OH). Suitable reporter molecules or labels, which may be used, include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, 5 magnetic particles, and the like.

Host cells transformed with nucleotide sequences encoding HPAK may be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein produced by a recombinant cell may be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression 10 vectors containing polynucleotides which encode HPAK may be designed to contain signal sequences which direct secretion of HPAK through a prokaryotic or eukaryotic cell membrane. Other recombinant constructions may be used to join sequences encoding HPAK to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides 15 such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, WA). The inclusion of cleavable linker sequences such as those specific for Factor XA or enterokinase (Invitrogen, San Diego, CA) between the purification domain and HPAK may be used to facilitate purification. 20 One such expression vector provides for expression of a fusion protein containing HPAK and a nucleic acid encoding 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography as described in Porath, J. et al. (1992, Prot. Exp. Purif. 3: 263-281) while the enterokinase cleavage site provides a means for purifying HPAK from the fusion protein. A 25 discussion of vectors which contain fusion proteins is provided in Kroll, D.J. et al. (1993; DNA Cell Biol. 12:441-453).

In addition to recombinant production, fragments of HPAK may be produced by direct peptide synthesis using solid-phase techniques Merrifield J. (1963) J. Am. Chem. Soc. 85:2149-2154). Protein synthesis may be performed using manual techniques or by automation. 30 Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Various fragments of HPAK may be chemically synthesized separately and combined using chemical methods to produce the full length molecule.

## THERAPEUTICS

Chemical and structural homology exists among HPAK, human pancreatic kallikrein, and african rat renal kallikrein. In addition, northern analysis (Figure 5) shows that six cDNA libraries containing HPAK transcripts are from prostate tissue. HPAK is believed to function in the prostate gland. Of the six prostate tissue libraries, five were from patients with prostate cancer. HPAK expression was also found in breast and parotid gland tissues from patients with tumors in these tissues. Thus, HPAK expression appears to be associated with the prostate gland and in the development of cancer.

Antagonists or inhibitors of HPAK may be used to suppress excessive cell proliferation. 10 Thus in one embodiment, antagonists or inhibitors of HPAK may be administered to a subject to treat or prevent cancer, including but not limited to, cancer of the prostate, parotid gland, and breast.

More specifically, antagonists or inhibitors of HPAK may be used to suppress excessive proliferation of prostate cells.

15 In other aspects, antibodies which are specific for HPAK may be used directly as an antagonist, or indirectly as a targeting or delivery mechanism for bringing a pharmaceutical agent to cells or tissue which express HPAK.

In another embodiment, a vector expressing antisense of the polynucleotide encoding HPAK may be administered to a subject to treat or prevent cancer. Examples of cancers include, 20 but are not limited to, cancer of the breast and parotid gland.

In another embodiment, a vector expressing antisense of the polynucleotide encoding HPAK may be administered to a subject to treat or prevent disorders of the prostate. Examples of such disorders of the prostate include, but are not limited to, prostate cancer and benign prostatic hyperplasia.

25 In other embodiments, any of the therapeutic proteins, antagonists, antibodies, agonists, antisense sequences or vectors described above may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy may be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to 30 effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

Antagonists or inhibitors of HPAK may be produced using methods which are generally known in the art. In particular, purified HPAK may be used to produce antibodies or to screen libraries of pharmaceutical agents to identify those which specifically bind HPAK.

The antibodies may be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and fragments produced by a Fab expression library. Neutralizing antibodies, (i.e., those which inhibit dimer formation) are especially preferred for therapeutic use.

For the production of antibodies, various hosts including goats, rabbits, rats, mice, humans, and others, may be immunized by injection with HPAK or any fragment or oligopeptide thereof which has immunogenic properties. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and Corynebacterium parvum are especially preferable.

It is preferred that the peptides, fragments, or oligopeptides used to induce antibodies to HPAK have an amino acid sequence consisting of at least five amino acids, and more preferably at least 10 amino acids. It is also preferable that they are identical to a portion of the amino acid sequence of the natural protein, and they may contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of HPAK amino acids may be fused with those of another protein such as keyhole limpet hemocyanin and antibody produced against the chimeric molecule.

Monoclonal antibodies to HPAK may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are 25 not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Kohler, G. et al. (1975) *Nature* 256:495-497; Kozbor, D. et al. (1985) *J. Immunol. Methods* 81:31-42; Cote, R.J. et al. (1983) *Proc. Natl. Acad. Sci.* 80:2026-2030; Cole, S.P. et al. (1984) *Mol. Cell Biol.* 62:109-120).

In addition, techniques developed for the production of "chimeric antibodies", the  
30 splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate  
antigen specificity and biological activity can be used (Morrison, S.L. et al. (1984) Proc. Natl.  
Acad. Sci. 81:6851-6855; Neuberger, M.S. et al. (1984) Nature 312:604-608; Takeda, S. et al.

(1985) *Nature* 314:452-454). Alternatively, techniques described for the production of single chain antibodies may be adapted, using methods known in the art, to produce HPAK-specific single chain antibodies. Antibodies with related specificity, but of distinct idiotypic composition, may be generated by chain shuffling from random combinatorial immunoglobulin libraries (Burton 5 D.R. (1991) *Proc. Natl. Acad. Sci.* 88:11120-3).

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature (Orlandi, R. et al. (1989) Proc. Natl. Acad. Sci. 86: 3833-3837; Winter, G. et al. (1991) Nature 349:293-299).

10 Antibody fragments which contain specific binding sites for HPAK may also be generated. For example, such fragments include, but are not limited to, the F(ab')2 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal 15 Fab fragments with the desired specificity (Huse, W.D. et al. (1989) Science 254:1275-1281).

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between 20 HPAK and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering HPAK epitopes is preferred, but a competitive binding assay may also be employed (Maddox, *supra*).

In another embodiment of the invention, the polynucleotides encoding HPAK, or any fragment thereof, or antisense molecules, may be used for therapeutic purposes. In one aspect, antisense to the polynucleotide encoding HPAK may be used in situations in which it would be desirable to block the transcription of the mRNA. In particular, cells may be transformed with sequences complementary to polynucleotides encoding HPAK. Thus, antisense molecules may be used to modulate HPAK activity, or to achieve regulation of gene function. Such technology is now well known in the art, and sense or antisense oligomers or larger fragments, can be designed from various locations along the coding or control regions of sequences encoding HPAK.

Expression vectors derived from retro viruses, adenovirus, herpes or vaccinia viruses, or

from various bacterial plasmids may be used for delivery of nucleotide sequences to the targeted organ, tissue or cell population. Methods which are well known to those skilled in the art can be used to construct recombinant vectors which will express antisense molecules complementary to the polynucleotides of the gene encoding HPAK. These techniques are described both in

5 Sambrook et al. (*supra*) and in Ausubel et al. (*supra*).

Genes encoding HPAK can be turned off by transforming a cell or tissue with expression vectors which express high levels of a polynucleotide or fragment thereof which encodes HPAK. Such constructs may be used to introduce untranslatable sense or antisense sequences into a cell. Even in the absence of integration into the DNA, such vectors may continue to transcribe RNA 10 molecules until they are disabled by endogenous nucleases. Transient expression may last for a month or more with a non-replicating vector and even longer if appropriate replication elements are part of the vector system.

As mentioned above, modifications of gene expression can be obtained by designing antisense molecules, DNA, RNA, or PNA, to the control regions of the gene encoding HPAK, 15 i.e., the promoters, enhancers, and introns. Oligonucleotides derived from the transcription initiation site, e.g., between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Recent therapeutic 20 advances using triplex DNA have been described in the literature (Gee, J.E. et al. (1994) In: Huber, B.E. and B.I. Carr, Molecular and Immunologic Approaches, Futura Publishing Co., Mt. Kisco, NY). The antisense molecules may also be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Ribozymes, enzymatic RNA molecules, may also be used to catalyze the specific 25 cleavage of RNA. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples which may be used include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of sequences encoding HPAK.

Specific ribozyme cleavage sites within any potential RNA target are initially identified 30 by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be

evaluated for secondary structural features which may render the oligonucleotide inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

Antisense molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding HPAK. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 or SP6. Alternatively, 10 these cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines, cells, or tissues.

RNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase 15 linkages within the backbone of the molecule. This concept is inherent in the production of PNAs and can be extended in all of these molecules by the inclusion of nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

20 Many methods for introducing vectors into cells or tissues are available and equally suitable for use *in vivo*, *in vitro*, and *ex vivo*. For *ex vivo* therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection and by liposome injections may be achieved using methods which are well known in the art.

25 Any of the therapeutic methods described above may be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

An additional embodiment of the invention relates to the administration of a pharmaceutical composition, in conjunction with a pharmaceutically acceptable carrier, for any of 30 the therapeutic effects discussed above. Such pharmaceutical compositions may consist of HPAK, antibodies to HPAK, mimetics, agonists, antagonists, or inhibitors of HPAK. The compositions may be administered alone or in combination with at least one other agent, such as

stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

5 The pharmaceutical compositions utilized in this invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain  
10 suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, PA).

15 Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

20 Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as 25 methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores may be used in conjunction with suitable coatings, such as concentrated  
30 sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for

product identification or to characterize the quantity of active compound, i.e., dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol.

Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or 5 starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations suitable for parenteral administration may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution.

10 Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or 15 triglycerides, or liposomes. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

20 The pharmaceutical compositions of the present invention may be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, 25 etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they can be placed in an 30 appropriate container and labeled for treatment of an indicated condition. For administration of HPAK, such labeling would include amount, frequency, and method of administration.

Pharmaceutical compositions suitable for use in the invention include compositions

wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in 5 cell culture assays, e.g., of neoplastic cells, or in animal models, usually mice, rabbits, dogs, or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective dose refers to that amount of active ingredient, for example 10 HPAK or fragments thereof, antibodies of HPAK, agonists, antagonists or inhibitors of HPAK, which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic 15 index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage 20 form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and 25 gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of 30 about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or

their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

## DIAGNOSTICS

In another embodiment, antibodies which specifically bind HPAK may be used for the diagnosis of conditions or diseases characterized by expression of HPAK, or in assays to monitor patients being treated with HPAK, agonists, antagonists or inhibitors. The antibodies useful for diagnostic purposes may be prepared in the same manner as those described above for therapeutics. Diagnostic assays for HPAK include methods which utilize the antibody and a label to detect HPAK in human body fluids or extracts of cells or tissues. The antibodies may be used with or without modification, and may be labeled by joining them, either covalently or non-covalently, with a reporter molecule. A wide variety of reporter molecules which are known in the art may be used, several of which are described above.

A variety of protocols including ELISA, RIA, and FACS for measuring HPAK are known in the art and provide a basis for diagnosing altered or abnormal levels of HPAK expression.

Normal or standard values for HPAK expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to HPAK under conditions suitable for complex formation. The amount of standard complex formation may be quantified by various methods, but preferably by photometric means. Quantities of HPAK expressed in subject, control and disease, samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

In another embodiment of the invention, the polynucleotides encoding HPAK may be used for diagnostic purposes. The polynucleotides which may be used include oligonucleotide sequences, antisense RNA and DNA molecules, and PNAs. The polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which expression of HPAK may be correlated with disease. The diagnostic assay may be used to distinguish between absence, presence, and excess expression of HPAK, and to monitor regulation of HPAK levels during therapeutic intervention.

In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding HPAK or closely related molecules, may be used to identify nucleic acid sequences which encode HPAK. The specificity of the probe, whether it is made from a highly specific region, e.g., 10 unique nucleotides in the

5' regulatory region, or a less specific region, e.g., especially in the 3' coding region, and the stringency of the hybridization or amplification (maximal, high, intermediate, or low) will determine whether the probe identifies only naturally occurring sequences encoding HPAK, alleles, or related sequences.

5        Probes may also be used for the detection of related sequences, and should preferably contain at least 50% of the nucleotides from any of the HPAK encoding sequences. The hybridization probes of the subject invention may be DNA or RNA and derived from the nucleotide sequence of SEQ ID NO:2 or from genomic sequence including promoter, enhancer elements, and introns of the naturally occurring HPAK.

10        Means for producing specific hybridization probes for DNAs encoding HPAK include the cloning of nucleic acid sequences encoding HPAK or HPAK derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, commercially available, and may be used to synthesize RNA probes *in vitro* by means of the addition of the appropriate RNA polymerases and the appropriate labeled nucleotides. Hybridization probes may be labeled by a 15 variety of reporter groups, for example, radionuclides such as 32P or 35S, or enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

20        Polynucleotide sequences encoding HPAK may be used for the diagnosis of conditions or diseases which are associated with expression of HPAK. Examples of such conditions or diseases include pancreatic disorders, asthma, and cancers of the prostate, parotid gland, and breast. The polynucleotide sequences encoding HPAK may be used in Southern or northern analysis, dot blot, or other membrane-based technologies; in PCR technologies; or in dip stick, pIN, ELISA or chip assays utilizing fluids or tissues from patient biopsies to detect altered HPAK expression. Such qualitative or quantitative methods are well known in the art.

25        In a particular aspect, the nucleotide sequences encoding HPAK may be useful in assays that detect activation or induction of various cancers, particularly those mentioned above. The nucleotide sequences encoding HPAK may be labeled by standard methods, and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantitated 30 and compared with a standard value. If the amount of signal in the biopsied or extracted sample is significantly altered from that of a comparable control sample, the nucleotide sequences have hybridized with nucleotide sequences in the sample, and the presence of altered levels of

nucleotide sequences encoding HPAK in the sample indicates the presence of the associated disease. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or in monitoring the treatment of an individual patient.

5        In order to provide a basis for the diagnosis of disease associated with expression of HPAK, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, which encodes HPAK, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the 10 values obtained from normal subjects with those from an experiment where a known amount of a substantially purified polynucleotide is used. Standard values obtained from normal samples may be compared with values obtained from samples from patients who are symptomatic for disease. Deviation between standard and subject values is used to establish the presence of disease.

15       Once disease is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to evaluate whether the level of expression in the patient begins to approximate that which is observed in the normal patient. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

20       With respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or 25 further progression of the cancer.

Additional diagnostic uses for oligonucleotides designed from the sequences encoding HPAK may involve the use of PCR. Such oligomers may be chemically synthesized, generated enzymatically, or produced from a recombinant source. Oligomers will preferably consist of two nucleotide sequences, one with sense orientation (5'->3') and another with antisense (3'<-5'), 30 employed under optimized conditions for identification of a specific gene or condition. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantitation of closely related DNA

or RNA sequences.

Methods which may also be used to quantitate the expression of HPAK include radiolabeling or biotinylating nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated (Melby, P.C. et al. (1993) J. 5 Immunol. Methods, 159:235-244; Duplaa, C. et al. (1993) Anal. Biochem. 229:236). The speed of quantitation of multiple samples may be accelerated by running the assay in an ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or colorimetric response gives rapid quantitation.

In another embodiment of the invention, the nucleic acid sequences which encode HPAK 10 may also be used to generate hybridization probes which are useful for mapping the naturally occurring genomic sequence. The sequences may be mapped to a particular chromosome or to a specific region of the chromosome using well known techniques. Such techniques include FISH, FACS, or artificial chromosome constructions, such as yeast artificial chromosomes, bacterial artificial chromosomes, bacterial P1 constructions or single chromosome cDNA libraries as 15 reviewed in Price, C.M. (1993) Blood Rev. 7:127-134, and Trask, B.J. (1991) Trends Genet. 7:149-154.

FISH (as described in Verma et al. (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York, NY) may be correlated with other physical 20 chromosome mapping techniques and genetic map data. Examples of genetic map data can be found in the 1994 Genome Issue of Science (265:1981f). Correlation between the location of the gene encoding HPAK on a physical chromosomal map and a specific disease, or predisposition to a specific disease, may help delimit the region of DNA associated with that genetic disease. The nucleotide sequences of the subject invention may be used to detect differences in gene 25 sequences between normal, carrier, or affected individuals.

25 In *situ* hybridization of chromosomal preparations and physical mapping techniques such as linkage analysis using established chromosomal markers may be used for extending genetic maps. Often the placement of a gene on the chromosome of another mammalian species, such as mouse, may reveal associated markers even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms, or parts 30 thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once the disease or syndrome has been crudely localized by genetic linkage to a particular genomic region, for

example. AT to 11q22-23 (Gatti, R.A. et al. (1988) *Nature* 336:577-580). any sequences mapping to that area may represent associated or regulatory genes for further investigation. The nucleotide sequence of the subject invention may also be used to detect differences in the chromosomal location due to translocation, inversion, etc. among normal, carrier, or affected individuals.

5 In another embodiment of the invention, HPAK, its catalytic or immunogenic fragments or oligopeptides thereof, can be used for screening libraries of compounds in any of a variety of drug screening techniques. The fragment employed in such screening may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The formation of binding complexes, between HPAK and the agent being tested, may be measured.

10 Another technique for drug screening which may be used provides for high throughput screening of compounds having suitable binding affinity to the protein of interest as described in published PCT application WO84/03564. In this method, as applied to HPAK large numbers of different small test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The test compounds are reacted with HPAK, or fragments thereof, and washed.

15 Bound HPAK is then detected by methods well known in the art. Purified HPAK can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

20 In another embodiment, one may use competitive drug screening assays in which neutralizing antibodies capable of binding HPAK specifically compete with a test compound for binding HPAK. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with HPAK.

25 In additional embodiments, the nucleotide sequences which encode HPAK may be used in any molecular biology techniques that have yet to be developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, including, but not limited to, such properties as the triplet genetic code and specific base pair interactions.

The examples below are provided to illustrate the subject invention and are not included for the purpose of limiting the invention.

30

## EXAMPLES

### I BRSTNOT05 cDNA Library Construction

The breast (BRSTNOT05) cDNA library was constructed from the breast tissue of a 58

year old Caucasian female. The patient was diagnosed with multicentric invasive grade 4 lobular carcinoma.

The frozen tissue was homogenized and lysed using a Brinkmann Homogenizer Polytron PT-3000 (Brinkmann Instruments, Westbury NJ) in guanidinium isothiocyanate solution. The lysate was centrifuged over a 5.7 M CsCl cushion using an Beckman SW28 rotor in a Beckman L8-70M Ultracentrifuge (Beckman Instruments) for 18 hours at 25,000 rpm at ambient temperature. The RNA was extracted with acid phenol pH 4.0, precipitated using 0.3 M sodium acetate and 2.5 volumes of ethanol, resuspended in RNase-free water and DNase treated at 37°C. Extraction and precipitation were repeated. The mRNA then isolated with the Qiagen Oligotex 10 kit (QIAGEN Inc; Chatsworth CA) and used to construct the cDNA library.

The mRNA was handled according to the recommended protocols in the SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (Cat. #18248-013; Gibco/BRL, Gaithersburg, MD). cDNAs were fractionated on a Sepharose CL4B column (Cat. #275105-01; Pharmacia), and those cDNAs exceeding 400 bp were ligated into pSport I. The plasmid pSport I 15 was subsequently transformed into DH5<sup>a</sup>™ competent cells (Cat. #18258-012; Gibco/BRL).

## II Isolation and Sequencing of cDNA Clones

Plasmid DNA was released from the cells and purified using the REAL Prep 96 Plasmid Kit (Catalog #26173; QIAGEN, Inc). This kit enables the simultaneous purification of 96 samples in a 96-well block using multi-channel reagent dispensers. The recommended protocol was employed except for the following changes: 1) the bacteria were cultured in 1 ml of sterile Terrific Broth (Catalog #22711, LIFE TECHNOLOGIES™, Gaithersburg, MD) with carbenicillin at 25 mg/L and glycerol at 0.4%; 2) after inoculation, the cultures were incubated for 19 hours and at the end of incubation, the cells were lysed with 0.3 ml of lysis buffer; and 3) following isopropanol precipitation, the plasmid DNA pellet was resuspended in 0.1 ml of distilled water. After the last step in the protocol, samples were transferred to a 96-well block for storage at 4° C.

The cDNAs were sequenced by the method of Sanger F. and A.R. Coulson (1975; J. Mol. Biol. 94:441f), using a Hamilton Micro Lab 2200 (Hamilton, Reno NV) in combination with 30 Peltier Thermal Cyclers (PTC200 from MJ Research, Watertown MA) and Applied Biosystems 377 DNA Sequencing Systems; and the reading frame was determined.

### III Homology Searching of cDNA Clones and Their Deded Proteins

The nucleotide sequences of the Sequence Listing or amino acid sequences deduced from them were used as query sequences against databases such as GenBank, SwissProt, BLOCKS, and Pima II. These databases which contain previously identified and annotated sequences were 5 searched for regions of homology (similarity) using BLAST, which stands for Basic Local Alignment Search Tool (Altschul (1993) supra, Altschul (1990) supra).

BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologs which may be of prokaryotic (bacterial) or 10 eukaryotic (animal, fungal or plant) origin. Other algorithms such as the one described in Smith R.F. and T.F. Smith (1992 Protein Engineering 5:35-51), incorporated herein by reference, can be used when dealing with primary sequence patterns and secondary structure gap penalties. As disclosed in this application, the sequences have lengths of at least 49 nucleotides, and no more than 12% uncalled bases (where N is recorded rather than A, C, G, or T).

15 The BLAST approach, as detailed in Karlin and Altschul (supra) and incorporated herein by reference, searches for matches between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. In this application, threshold was set at  $10^{-25}$  for nucleotides and  $10^{-14}$  for peptides.

20 Incyte nucleotide sequences were searched against the GenBank databases for primate (pri), rodent (rod), and mammalian sequences (mamm), and deduced amino acid sequences from the same clones are searched against GenBank functional protein databases, mammalian (mamp), vertebrate (vrtp) and eukaryote (eukp), for homology. The relevant database for a particular match were reported as a GIxxx±p (where xxx is pri, rod, etc and if present, p = peptide). The 25 product score is calculated as follows: the % nucleotide or amino acid identity [between the query and reference sequences] in BLAST is multiplied by the % maximum possible BLAST score [based on the lengths of query and reference sequences] and then divided by 100. Where an Incyte Clone was homologous to several sequences, up to five matches were provided with their relevant scores. In an analogy to the hybridization procedures used in the laboratory, the 30 electronic stringency for an exact match was set at 70, and the conservative lower limit for an exact match was set at approximately 40 (with 1-2% error due to uncalled bases).

**IV Northern Analysis**

Northern analysis is a laboratory technique used to detect the presence of a transcript of a gene and involves the hybridization of a labeled nucleotide sequence to a membrane on which RNAs from a particular cell type or tissue have been bound (Sambrook et al., *supra*).

5 Analogous computer techniques using BLAST (Altschul, S.F. 1993 and 1990, *supra*) are used to search for identical or related molecules in nucleotide databases such as GenBank or the LIFESEQ™ database (Incyte Pharmaceuticals). This analysis is much faster than multiple membrane-based hybridizations. In addition, the sensitivity of the computer search can be modified to determine whether any particular match is categorized as exact or homologous.

10 The basis of the search is the product score which is defined as:

$$\frac{\% \text{ sequence identity} \times \% \text{ maximum BLAST score}}{100}$$

The product score takes into account both the degree of similarity between two sequences and the length of the sequence match. For example, with a product score of 40, the match will be exact 15 within a 1-2% error; and at 70, the match will be exact. Homologous molecules are usually identified by selecting those which show product scores between 15 and 40, although lower scores may identify related molecules.

The results of northern analysis are reported as a list of libraries in which the transcript encoding HPAK occurs. Abundance and percent abundance are also reported. Abundance 20 directly reflects the number of times a particular transcript is represented in a cDNA library, and percent abundance is abundance divided by the total number of sequences examined in the cDNA library.

25 **V Extension of HPAK-Encoding Polynucleotides to Full Length or to Recover Regulatory Sequences**

Full length HPAK-encoding nucleic acid sequence (SEQ ID NO:2) is used to design oligonucleotide primers for extending a partial nucleotide sequence to full length or for obtaining 5' or 3', intron or other control sequences from genomic libraries. One primer is synthesized to initiate extension in the antisense direction (XLR) and the other is synthesized to extend sequence 30 in the sense direction (XLF). Primers are used to facilitate the extension of the known sequence "outward" generating amplicons containing new, unknown nucleotide sequence for the region of interest. The initial primers are designed from the cDNA using OLIGO 4.06 (National

Biosciences), or another appropriate program, to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68°-72° C. Any stretch of nucleotides which would result in hairpin structures and primer-primer dimerizations is avoided.

5 The original, selected cDNA libraries, or a human genomic library are used to extend the sequence: the latter is most useful to obtain 5' upstream regions. If more extension is necessary or desired, additional sets of primers are designed to further extend the known region.

By following the instructions for the XL-PCR kit (Perkin Elmer) and thoroughly mixing the enzyme and reaction mix, high fidelity amplification is obtained. Beginning with 40 pmol of 10 each primer and the recommended concentrations of all other components of the kit, PCR is performed using the Peltier Thermal Cycler (PTC200; M.J. Research, Watertown, MA) and the following parameters:

	Step 1	94° C for 1 min (initial denaturation)
	Step 2	65° C for 1 min
15	Step 3	68° C for 6 min
	Step 4	94° C for 15 sec
	Step 5	65° C for 1 min
	Step 6	68° C for 7 min
	Step 7	Repeat step 4-6 for 15 additional cycles
20	Step 8	94° C for 15 sec
	Step 9	65° C for 1 min
	Step 10	68° C for 7:15 min
	Step 11	Repeat step 8-10 for 12 cycles
	Step 12	72° C for 8 min
25	Step 13	4° C (and holding)

A 5-10  $\mu$ l aliquot of the reaction mixture is analyzed by electrophoresis on a low concentration (about 0.6-0.8%) agarose mini-gel to determine which reactions were successful in extending the sequence. Bands thought to contain the largest products are selected and removed 30 from the gel. Further purification involves using a commercial gel extraction method such as QIAQuick™ (QIAGEN Inc., Chatsworth, CA). After recovery of the DNA, Klenow enzyme is used to trim single-stranded, nucleotide overhangs creating blunt ends which facilitate religation and cloning.

After ethanol precipitation, the products are redissolved in 13  $\mu$ l of ligation buffer, 1  $\mu$ l 35 T4-DNA ligase (15 units) and 1  $\mu$ l T4 polynucleotide kinase are added, and the mixture is incubated at room temperature for 2-3 hours or overnight at 16° C. Competent E. coli cells (in

40  $\mu$ l of appropriate media are transformed with 3  $\mu$ l of ligation mixture and cultured in 80  $\mu$ l of SOC medium (Sambrook et al., supra). After incubation for one hour at 37° C, the whole transformation mixture is plated on Luria Bertani (LB)-agar (Sambrook et al., supra) containing 2x Carb. The following day, several colonies are randomly picked from each plate and cultured 5 in 150  $\mu$ l of liquid LB/2x Carb medium placed in an individual well of an appropriate, commercially-available, sterile 96-well microtiter plate. The following day, 5  $\mu$ l of each overnight culture is transferred into a non-sterile 96-well plate and after dilution 1:10 with water, 5  $\mu$ l of each sample is transferred into a PCR array.

For PCR amplification, 18  $\mu$ l of concentrated PCR reaction mix (3.3x) containing 4 units 10 of rTth DNA polymerase, a vector primer, and one or both of the gene specific primers used for the extension reaction are added to each well. Amplification is performed using the following conditions:

	Step 1	94° C for 60 sec
	Step 2	94° C for 20 sec
15	Step 3	55° C for 30 sec
	Step 4	72° C for 90 sec
	Step 5	Repeat steps 2-4 for an additional 29 cycles
	Step 6	72° C for 180 sec
	Step 7	4° C (and holding)

20 Aliquots of the PCR reactions are run on agarose gels together with molecular weight markers. The sizes of the PCR products are compared to the original partial cDNAs, and appropriate clones are selected, ligated into plasmid, and sequenced.

## VI Labeling and Use of Hybridization Probes

Hybridization probes derived from SEQ ID NO:2 are employed to screen cDNAs. 25 genomic DNAs, or mRNAs. Although the labeling of oligonucleotides, consisting of about 20 base-pairs, is specifically described, essentially the same procedure is used with larger cDNA fragments. Oligonucleotides are designed using state-of-the-art software such as OLIGO 4.06 (National Biosciences), labeled by combining 50 pmol of each oligomer and 250  $\mu$ Ci of [ $\gamma$ - $^{32}$ P] adenosine triphosphate (Amersham) and T4 polynucleotide kinase (DuPont NEN®, Boston, MA). 30 The labeled oligonucleotides are substantially purified with Sephadex G-25 superfine resin column (Pharmacia & Upjohn). A portion containing 10<sup>7</sup> counts per minute of each of the sense and antisense oligonucleotides is used in a typical membrane based hybridization analysis of human genomic DNA digested with one of the following endonucleases (Ase I, Bgl II, Eco RI, Pst I, Xba I, or Pvu II; DuPont NEN®).

The DNA from each digest is fractionated on a 0.7 percent agarose gel and transferred to nylon membranes (Nytran Plus, Schleicher & Schuell, Durham, NH). Hybridization is carried out for 16 hours at 40°C. To remove nonspecific signals, blots are sequentially washed at room temperature under increasingly stringent conditions up to 0.1 x saline sodium citrate and 0.5% sodium dodecyl sulfate. After XOMAT ART™ film (Kodak, Rochester, NY) is exposed to the blots in a Phosphoimager cassette (Molecular Dynamics, Sunnyvale, CA) for several hours, hybridization patterns are compared visually.

## VII Antisense Molecules

Antisense molecules to the HPAK-encoding sequence, or any part thereof, is used to 10 inhibit *in vivo* or *in vitro* expression of naturally occurring HPAK. Although use of antisense oligonucleotides, comprising about 20 base-pairs, is specifically described, essentially the same procedure is used with larger cDNA fragments. An oligonucleotide based on the coding sequences of HPAK, as shown in Figures 1A, 1B and 1C, is used to inhibit expression of naturally occurring HPAK. The complementary oligonucleotide is designed from the most 15 unique 5' sequence as shown in Figures 1A, 1B and 1C and used either to inhibit transcription by preventing promoter binding to the upstream nontranslated sequence or translation of an HPAK-encoding transcript by preventing the ribosome from binding. Using an appropriate portion of the signal and 5' sequence of SEQ ID NO:2, an effective antisense oligonucleotide includes any 15-20 nucleotides spanning the region which translates into the signal or 5' coding sequence of 20 the polypeptide as shown in Figures 1A, 1B and 1C.

## VIII Expression of HPAK

Expression of HPAK is accomplished by subcloning the cDNAs into appropriate vectors and transforming the vectors into host cells. In this case, the cloning vector, pSport, previously used for the generation of the cDNA library is used to express HPAK in *E. coli*. Upstream of the 25 cloning site, this vector contains a promoter for β-galactosidase, followed by sequence containing the amino-terminal Met, and the subsequent seven residues of β-galactosidase. Immediately following these eight residues is a bacteriophage promoter useful for transcription and a linker containing a number of unique restriction sites.

Induction of an isolated, transformed bacterial strain with IPTG using standard methods 30 produces a fusion protein which consists of the first eight residues of β-galactosidase, about 5 to 15 residues of linker, and the full length protein. The signal residues direct the secretion of HPAK into the bacterial growth media which can be used directly in the following assay for

activity

#### IX Demonstration of HPAK Activity

HPAK's proteolytic activity can be determined by methods described by Christensson A. et al. (1990, Eur. J. Biochem. 194: 755-763). Substrates for proteolytic cleavage are found in 5 human semen. Human seminal plasma is collected and coagulated semen is washed free of soluble components. HPAK is incubated with coagulated semen in a buffer consisting of 50 mmol/l TRIS-HCl pH 7.8, with 0.1 mol/l NaCl. Reactions are performed at 37 °C. After 10 incubation periods of different durations (from 0 to 30 minutes) the samples are analyzed by SDS-PAGE. The resulting pattern of peptide fragments is compared and quantitated using a control sample in which HPAK is not added.

#### X Production of HPAK Specific Antibodies

HPAK that is substantially purified using PAGE electrophoresis (Sambrook, *supra*), or other purification techniques, is used to immunize rabbits and to produce antibodies using standard protocols. The amino acid sequence deduced from SEQ ID NO:2 is analyzed using 15 DNASTAR software (DNASTAR Inc) to determine regions of high immunogenicity and a corresponding oligopeptide is synthesized and used to raise antibodies by means known to those of skill in the art. Selection of appropriate epitopes, such as those near the C-terminus or in hydrophilic regions, is described by Ausubel et al. (*supra*), and others.

Typically, the oligopeptides are 15 residues in length, synthesized using an Applied 20 Biosystems Peptide Synthesizer Model 431A using fmoc-chemistry, and coupled to keyhole limpet hemocyanin (KLH, Sigma, St. Louis, MO) by reaction with N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS: Ausubel et al., *supra*). Rabbits are immunized with the oligopeptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for 25 anti-peptide activity, for example, by binding the peptide to plastic, blocking with 1% BSA, reacting with rabbit antisera, washing, and reacting with radioiodinated, goat anti-rabbit IgG.

#### XI Purification of Naturally Occurring HPAK Using Specific Antibodies

Naturally occurring or recombinant HPAK is substantially purified by immunoaffinity chromatography using antibodies specific for HPAK. An immunoaffinity column is constructed by covalently coupling HPAK antibody to an activated chromatographic resin, such as 30 CnBr-activated Sepharose (Pharmacia & Upjohn). After the coupling, the resin is blocked and washed according to the manufacturer's instructions.

Media containing HPAK is passed over the immunoaffinity column, and the column is

washed under conditions that allow the preferential absorbance of HPAK (e.g., high ionic strength buffers in the presence of detergent). The column is eluted under conditions that disrupt antibody/HPAK binding (e.g., a buffer of pH 2-3 or a high concentration of a chaotropic, such as urea or thiocyanate ion), and HPAK is collected.

## 5 XII Identification of Molecules Which Interact with HPAK

HPAK or biologically active fragments thereof are labeled with  $^{125}\text{I}$  Bolton-Hunter reagent (Bolton et al. (1973) Biochem. J. 133: 529). Candidate molecules previously arrayed in the wells of a multi-well plate are incubated with the labeled HPAK, washed and any wells with labeled HPAK complex are assayed. Data obtained using different concentrations of HPAK are 10 used to calculate values for the number, affinity, and association of HPAK with the candidate molecules.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit 15 of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.



Phe Asp Asp Glu Asn Thr Ala Gln Phe Val His Val Ser Glu Ser Phe  
 85 90 95  
 Pro His Pro Gly Phe Asn Met Ser Leu Leu Glu Asn His Thr Arg Gln  
 100 105 110  
 Ala Asp Glu Asp Tyr Ser His Asp Leu Met Leu Leu Arg Leu Thr Glu  
 115 120 125  
 Pro Ala Asp Thr Ile Thr Asp Ala Val Lys Val Val Glu Leu Pro Thr  
 130 135 140  
 Gln Glu Pro Glu Val Gly Ser Thr Cys Leu Al Ser Gly Trp Gly Ser  
 145 150 155 160  
 Ile Glu Pro Glu Asn Phe Ser Phe Pro Asp Asp Leu Gln Cys Val Asp  
 165 170 175  
 Leu Lys Ile Leu Pro Asn Asp Glu Cys Glu Lys Ala His Val Gln Lys  
 180 185 190  
 Val Thr Asp Phe Met Leu Cys Val Gly His Leu Glu Gly Gly Lys Asp  
 195 200 205  
 Thr Cys Val Gly Asp Ser Gly Gly Pro Leu Met Cys Asp Gly Val Leu  
 210 215 220  
 Gln Gly Val Thr Ser Trp Gly Tyr Val Pro Cys Gly Thr Pro Asn Lys  
 225 230 235 240  
 Pro Ser Val Ala Val Arg Val Leu Ser Tyr Val Lys Trp Ile Glu Asp  
 245 250 255  
 Thr Ile Ala Glu Asn Ser  
 260

## (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 833 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTGGCCCT	GGACACCTCT	GTCACCATGT	GGTTCTGGT	TCTGTGCC	GCCCTGTCCC	60
TGGGGGGGAC	TGGTGTGCG	CCCCCGATTC	AGTCCCCGAT	TGTGGGAGGC	TGGGAGTGTG	120
AGCAGCATT	CCAGCCTGG	CAGGCGGCTC	TGTACCAGAA	GACGCCGCTA	CTCTGTGGG	180
CGACGNTCA	NGCCCCAGA	TGGTCTCTGA	CAGCAGGCCA	CTGCTCTNAAG	CCCCGCTACA	240
TAGTTCACCT	GGGGCAGCAC	AACTCCAGA	AGGAGGGAGGG	CTGTGAGCAG	ACCCGGACAG	300
CCACTGAGTC	CTTCCCCCAC	CCCGGCTTCA	ACAACAGCCT	CCCCAACAAA	GACCACCGCA	360
ATGACATCAT	GCTGGTGAAG	ATGGCATCGC	CAGTCTCCAT	CACCTGGGCT	GTGCGACCCC	420
TCACCCCTCTC	CTCACGCTGT	GTCACTGCTG	GCACCAAGCTG	CCTCATTTCC	GGCTGGGCA	480
GCACGTCCAG	CCCCCAGTTA	CGCCTGCC	ACACCTGCG	ATGCGCCAAC	ATCACCATCA	540
TTGAGCACCA	GAAGTGTGAG	AACGCCCTACC	CCGGCAACAT	CACAGACACC	ATGGTGTGTG	600
CCAGCGTGC	GGAAAGGGGGC	AAGGACTCCT	GCCAGGGTGA	CTCCGGGGGC	CCTCTGGTCT	660
GTAACCAGTC	TCTTCAAGGC	ATTATCTCCT	GGGGCCAGGA	TCCGTGTGCG	ATCACCCGAA	720
AGCCTGGTGT	CTACACGAAA	GTCTGCAAAT	ATGTGGACTG	GATCCAGGAG	ACGATGAAGA	780
ACAATTAGAC	TGGACNTCAC	CTCCGAANCC	CCCACAGCCC	ATCACCCCTCC	ATT	833

## (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 262 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: GenBank
- (B) CLONE: 186653

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Trp Phe Leu Val Leu Cys Leu Ala Leu Ser Leu Gly Gly Thr Gly  
 1 5 10 15  
 Ala Ala Pro Pro Ile Gin Ser Arg Ile Val Gly Gly Trp Glu Cys Glu  
 20 25 30  
 Gin His Ser Gin Pro Trp Gln Ala Ala Leu Tyr His Phe Ser Thr Phe  
 35 40 45  
 Gln Cys Gly Gly Ile Leu Val His Arg Gln Trp Val Leu Thr Ala Ala  
 50 55 60  
 His Cys Ile Ser Asp Asn Tyr Gln Leu Trp Leu Gly Arg His Asn Leu  
 65 70 75 80  
 Phe Asp Asp Glu Asn Thr Ala Gln Phe Val His Val Ser Glu Ser Phe  
 85 90 95  
 Pro His Pro Gly Phe Asn Met Ser Leu Leu Glu Asn His Thr Arg Gln  
 100 105 110  
 Ala Asp Glu Asp Tyr Ser His Asp Leu Met Leu Leu Arg Leu Thr Glu  
 115 120 125  
 Pro Ala Asp Thr Ile Thr Asp Ala Val Lys Val Val Glu Leu Pro Thr  
 130 135 140  
 Gln Glu Pro Glu Val Gly Ser Thr Cys Leu Ala Ser Gly Trp Gly Ser  
 145 150 155 160  
 Ile Glu Pro Glu Asn Phe Ser Phe Pro Asp Asp Leu Gln Cys Val Asp  
 165 170 175  
 Leu Lys Ile Leu Pro Asn Asp Glu Cys Glu Lys Ala His Val Gln Lys  
 180 185 190  
 Val Thr Asp Phe Met Leu Cys Val Gly His Leu Glu Gly Gly Lys Asp  
 195 200 205  
 Thr Cys Val Gly Asp Ser Gly Gly Pro Leu Met Cys Asp Gly Val Leu  
 210 215 220  
 Gln Gly Val Thr Ser Trp Gly Tyr Val Pro Cys Gly Thr Pro Asn Lys  
 225 230 235 240  
 Pro Ser Val Ala Val Arg Val Leu Ser Tyr Val Lys Trp Ile Glu Asp  
 245 250 255  
 Thr Ile Ala Glu Asn Ser  
 260

## (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 263 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: GenBank
- (B) CLONE: 55527

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Trp Phe Leu Ile Leu Phe Leu Ala Leu Phe Leu Gly Gly Ile Asp  
 1 5 10 15  
 Ala Ala Pro Pro Val Gln Ser Arg Ile Ile Gly Gly Phe Asn Cys Glu  
 20 25 30  
 Lys Asn Ser Gln Pro Trp His Val Ala Val Tyr Arg Phe Ala Arg Tyr  
 35 40 45  
 Gln Cys Gly Gly Val Leu Leu Asp Ala Asn Trp Val Leu Thr Ala Ala  
 50 55 60  
 His Cys Tyr Asn Asp Lys Tyr Gln Val Trp Leu Gly Lys Asn Asn Arg  
 65 70 75 80  
 Phe Glu Asp Glu Pro Ser Ala Gln His Gln Leu Ile Ser Lys Ala Ile  
 85 90 95  
 Pro His Pro Gly Phe Asn Met Ser Leu Leu Asn Lys Asp His Thr Pro  
 100 105 110  
 His Pro Glu Asp Asp Tyr Ser Asn Asp Leu Met Leu Val Arg Leu Lys

115	120	125
Lys Pro Ala Glu Ile Thr Asp Val Val Lys Pro Ile Asp Leu Pro Thr		
130	135	140
Glu Glu Pro Thr Val Gly Ser Arg Cys Leu Ala Ser Gly Trp Gly Ser		
145	150	155
160		
Thr Thr Pro Thr Glu Glu Phe Glu Tyr Ser His Asp Leu Gln Cys Val		
165	170	175
Tyr Leu Glu Leu Leu Ser Asn Glu Val Cys Ala Lys Ala His Thr Glu		
180	185	190
Lys Val Thr Asp Thr Met Leu Cys Ala Gly Glu Met Asp Gly Gly Lys		
195	200	205
Asp Thr Cys Val Gly Asp Ser Gly Gly Pro Leu Ile Cys Asp Gly Val		
210	215	220
Leu Gln Gly Ile Thr Ser Trp Gly Pro Thr Pro Cys Ala Leu Pro Asn		
225	230	235
240		
Val Pro Gly Ile Tyr Thr Lys Leu Ile Glu Tyr Arg Ser Trp Ile Lys		
245	250	255
Asp Val Met Ala Asn Asn Pro		
260		

What is claimed is:

1. A substantially purified prostate-associated kallikrein protein comprising the amino acid sequence of SEQ ID NO:1 or fragments thereof.
2. An isolated and purified polynucleotide sequence encoding the prostate-associated kallikrein protein of claim 1.
3. A polynucleotide sequence which hybridizes under stringent conditions to the polynucleotide sequence of claim 2.
4. A hybridization probe comprising the polynucleotide sequence of claim 2.
5. An isolated and purified polynucleotide sequence comprising SEQ ID NO:2 or variants thereof.
6. A polynucleotide sequence which is complementary to the polynucleotide sequence of claim 2 or variants thereof.
7. A hybridization probe comprising the polynucleotide sequence of claim 6.
8. An expression vector containing the polynucleotide sequence of claim 2.
- 15 9. A host cell containing the vector of claim 8.
10. A method for producing a polypeptide comprising the amino acid sequence of SEQ ID NO:1 the method comprising the steps of:
  - a) culturing the host cell of claim 9 under conditions suitable for the expression of the polypeptide; and
  - 20 b) recovering the polypeptide from the host cell culture.
11. A purified antibody which binds specifically to the polypeptide of claim 1.
12. A purified agonist which specifically binds to and modulates the activity of the polypeptide of claim 1.
13. A purified antagonist which specifically binds to and modulates the activity of the 25 polypeptide of claim 1.
14. A pharmaceutical composition comprising a substantially purified antagonist of claim 13 in conjunction with a suitable pharmaceutical carrier.
15. A method for treating cancer comprising administering to a subject in need of such treatment an effective amount of the pharmaceutical composition of claim 14.
- 30 16. A method for treating disorders of the prostate comprising administering to a subject in need of such treatment an effective amount of the pharmaceutical composition of claim 14.

17. A method for detection of polynucleotides encoding kallikrein in a biological sample comprising the steps of:

- a) hybridizing the polynucleotide of claim 6 to nucleic acid material of a biological sample, thereby forming a hybridization complex; and
- 5 b) detecting said hybridization complex, wherein the presence of said complex correlates with the presence of a polynucleotide encoding kallikrein in said biological sample.

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FIGURE 1A

ATG	GCA	TCG	CCA	GTC	TCC	ATC	ACC	TGG	GCT	GTC	CGA	CCC	CTC	ACC	CTC	TCC	TCA	434	
M	A	S	P	V	S	I	T	W	A	V	R	P	L	T	L	S	S		
443			452			461			470			479			488				
CGC	TGT	GTC	ACT	GCT	GGC	ACC	AGC	TGC	CTC	ATT	TCC	GGC	TGG	GGC	AGC	ACG	TCC		
R	C	V	T	A	G	T	S	C	L	I	S	G	W	G	S	T	S		
497			506			515			524			533			542				
AGC	CCC	CAG	TTA	CGC	CTG	CCT	CAC	ACC	TTG	CGA	TGG	GCC	AAC	ATC	ACC	ATC	ATT		
S	P	Q	L	R	L	P	H	T	L	R	C	A	N	I	T	I	I		
551			560			569			578			587			596				
GAG	CAC	CAG	AAG	TGT	GAG	AAC	GCC	TAC	CCC	GGC	AAC	ATC	ACA	GAC	ACC	ATG	GTC		
E	H	Q	K	C	E	N	A	Y	P	G	N	I	T	D	T	M	V		
605			614			623			632			641			650				
TGT	GCC	AGC	GTG	CAG	GAA	GGG	GGC	AAG	GAC	TCC	TGC	CAG	GGT	GAC	TCC	GGG	GGC		
C	A	S	V	Q	E	G	G	K	D	S	C	Q	G	D	S	G	G		
659			668			677			686			695			704				
CCT	CTG	GTC	TGT	AAC	CAG	TCT	CTT	CAA	GGC	ATT	ATC	TCC	TGG	GGC	CAG	GAT	CCG		
P	L	V	C	N	Q	S	L	Q	G	I	I	S	W	G	Q	D	P		
713			722			731			740			749			758				
TGT	GCG	ATC	ACC	CGA	AAG	CCT	GGT	GTC	TAC	ACG	AAA	GTC	TGC	AAA	TAT	GTG	GAC		
C	A	I	T	R	K	P	G	V	Y	T	K	V	C	K	Y	V	D		

FIGURE 1B

FIGURE 1C

1 [M W F L V L C L A L S L G G T G A A P P I Q S R I V G G W E] SEQ ID NO-1

1 [M W F L V L C L A L S L G G T G A A P P I Q S R I V G G W E] GI 186653

1 [M W F L] I [L] F [L A L] F [L G G] I D A A P P V Q S R I T G G F N GI 55527

31 [C E Q H S Q P W Q A A L Y Q K T R L L C G A T X X A P R W F] SEQ ID NO-1

31 [C E Q H S Q P W Q A A L Y H F S T F Q C G G I L V H R Q W V] GI 186653

31 [C E K N S Q P W H V A V Y R F A R Y Q C G G V I L D A N W V] GI 55527

61 [L T A A H C L K P R Y I V H L G Q H N L Q K E E G C E Q T R] SEQ ID NO-1

61 [L T A A H C I S D N Y Q L W L G R H N L F D D E N T A Q F V] GI 186653

61 [L T A A H C Y N D K Y Q V W L G K N N R E E D E P S A Q H Q] GI 55527

91 T A T E S F P H P G F N N S L P N K D H - - - - - R N] SEQ ID NO-1

91 H V S E S F P H P G F N M S L L E - N H T R Q A D E D Y S H GI 186653

91 L I S K A I P H P G F N M S L L N K D H T P H P E D D Y S N] GI 55527

113 D I M L V K M A S P V - S I T W A V R P L T L S S R C V T A SEQ ID NO-1

120 D L M L L R L T E P A D T I T D A V K V V E L P T Q E P E V] GI 186653

121 D L M L V R L K K P A E - I T D V V K P I D L P T E E P T V] GI 55527

FIGURE 2A

142  $\boxed{G} \boxed{T} \boxed{S} \boxed{C} \boxed{L} \boxed{I} \boxed{S} \boxed{G} \boxed{W} \boxed{G} \boxed{S} \boxed{T} \boxed{S} \boxed{S} \boxed{P} \boxed{Q} - \boxed{L} \boxed{R} \boxed{L} \boxed{P} \boxed{H} \boxed{T} \boxed{L} \boxed{R} \boxed{C} \boxed{A} \boxed{N} \boxed{I} \boxed{T}$  SEQ ID NO-1  
 150  $\boxed{G} \boxed{S} \boxed{T} \boxed{C} \boxed{L} \boxed{A} \boxed{S} \boxed{G} \boxed{W} \boxed{G} \boxed{S} \boxed{I} \boxed{E} \boxed{P} - \boxed{E} \boxed{N} \boxed{F} \boxed{S} \boxed{F} \boxed{P} \boxed{D} \boxed{D} \boxed{L} \boxed{Q} \boxed{C} \boxed{V} \boxed{D} \boxed{L} \boxed{K}$  GI 186653  
 150  $\boxed{G} \boxed{S} \boxed{R} \boxed{C} \boxed{L} \boxed{A} \boxed{S} \boxed{G} \boxed{W} \boxed{G} \boxed{S} \boxed{T} \boxed{T} \boxed{P} \boxed{E} \boxed{F} \boxed{Y} \boxed{S} \boxed{H} \boxed{D} \boxed{L} \boxed{Q} \boxed{C} \boxed{V} \boxed{Y} \boxed{L} \boxed{E}$  GI 55527

171  $\boxed{I} \boxed{I} \boxed{E} \boxed{H} \boxed{Q} \boxed{K} \boxed{C} \boxed{E} \boxed{N} \boxed{A} \boxed{Y} \boxed{P} \boxed{G} \boxed{N} \boxed{I} \boxed{T} \boxed{D} \boxed{T} \boxed{M} \boxed{V} \boxed{C} \boxed{A} \boxed{S} \boxed{V} \boxed{Q} \boxed{E} \boxed{G} \boxed{G} \boxed{K} \boxed{D}$  SEQ ID NO-1  
 179  $\boxed{I} \boxed{L} \boxed{P} \boxed{N} \boxed{D} \boxed{E} \boxed{C} \boxed{E} \boxed{K} \boxed{A} \boxed{H} \boxed{V} \boxed{Q} \boxed{K} \boxed{V} \boxed{T} \boxed{D} \boxed{E} \boxed{M} \boxed{L} \boxed{C} \boxed{V} \boxed{G} \boxed{H} \boxed{L} \boxed{E} \boxed{G} \boxed{G} \boxed{K} \boxed{D}$  GI 186653  
 180  $\boxed{L} \boxed{L} \boxed{S} \boxed{N} \boxed{E} \boxed{V} \boxed{C} \boxed{A} \boxed{K} \boxed{A} \boxed{H} \boxed{T} \boxed{E} \boxed{K} \boxed{V} \boxed{T} \boxed{D} \boxed{T} \boxed{M} \boxed{L} \boxed{C} \boxed{A} \boxed{G} \boxed{E} \boxed{M} \boxed{D} \boxed{G} \boxed{G} \boxed{K} \boxed{D}$  GI 55527

201  $\boxed{S} \boxed{C} \boxed{Q} \boxed{G} \boxed{D} \boxed{S} \boxed{G} \boxed{G} \boxed{P} \boxed{L} \boxed{V} \boxed{C} \boxed{N} \boxed{Q} \boxed{S} \boxed{L} \boxed{Q} \boxed{G} \boxed{I} \boxed{I} \boxed{S} \boxed{W} \boxed{G} \boxed{Q} \boxed{D} \boxed{P} \boxed{C} \boxed{A} \boxed{I} \boxed{T}$  SEQ ID NO-1  
 209  $\boxed{T} \boxed{C} \boxed{V} \boxed{G} \boxed{D} \boxed{S} \boxed{G} \boxed{G} \boxed{P} \boxed{L} \boxed{M} \boxed{C} \boxed{D} \boxed{G} \boxed{V} \boxed{L} \boxed{Q} \boxed{G} \boxed{V} \boxed{T} \boxed{S} \boxed{W} \boxed{G} \boxed{Y} \boxed{V} \boxed{P} \boxed{C} \boxed{G} \boxed{T} \boxed{P}$  GI 186653  
 210  $\boxed{T} \boxed{C} \boxed{V} \boxed{G} \boxed{D} \boxed{S} \boxed{G} \boxed{G} \boxed{P} \boxed{L} \boxed{I} \boxed{C} \boxed{D} \boxed{G} \boxed{V} \boxed{L} \boxed{Q} \boxed{G} \boxed{I} \boxed{T} \boxed{S} \boxed{W} \boxed{G} \boxed{P} \boxed{T} \boxed{P} \boxed{C} \boxed{A} \boxed{L} \boxed{P}$  GI 55527

231  $\boxed{R} \boxed{K} \boxed{P} \boxed{G} \boxed{V} \boxed{Y} \boxed{T} \boxed{K} \boxed{V} \boxed{C} \boxed{K} \boxed{Y} \boxed{V} \boxed{D} \boxed{W} \boxed{I} \boxed{Q} \boxed{E} \boxed{T} \boxed{M} \boxed{K} \boxed{N} \boxed{N}$  S  
 239  $\boxed{N} \boxed{K} \boxed{P} \boxed{S} \boxed{V} \boxed{A} \boxed{V} \boxed{R} \boxed{V} \boxed{L} \boxed{S} \boxed{Y} \boxed{V} \boxed{K} \boxed{W} \boxed{I} \boxed{E} \boxed{D} \boxed{T} \boxed{I} \boxed{A} \boxed{E} \boxed{N}$  P  
 240  $\boxed{N} \boxed{V} \boxed{P} \boxed{G} \boxed{I} \boxed{Y} \boxed{T} \boxed{K} \boxed{L} \boxed{I} \boxed{E} \boxed{Y} \boxed{R} \boxed{S} \boxed{W} \boxed{I} \boxed{K} \boxed{D} \boxed{V} \boxed{M} \boxed{A} \boxed{N} \boxed{N}$  P

FIGURE 2B

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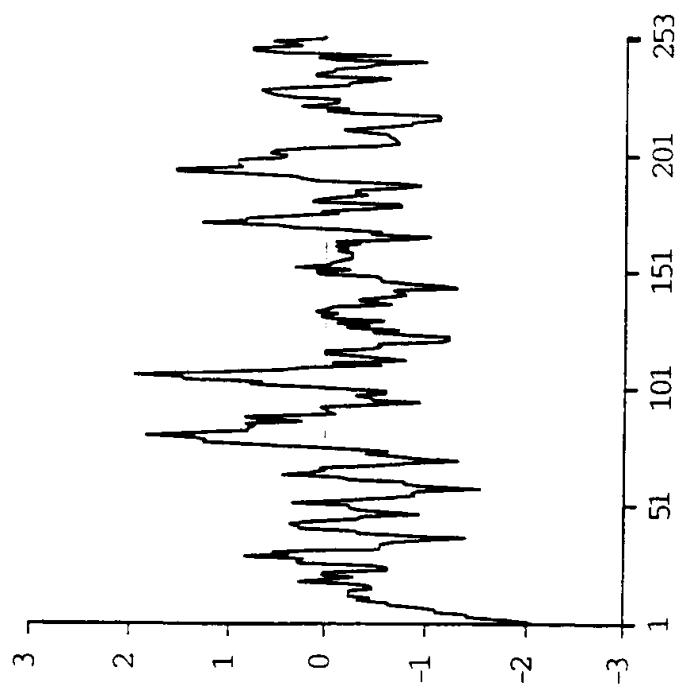


FIGURE 3

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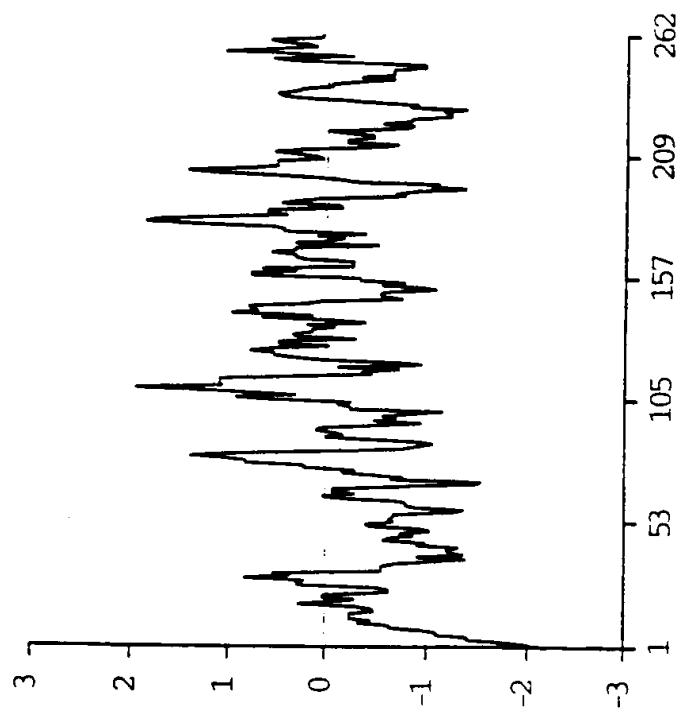


FIGURE 4

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Library	Lib Description	Abun	Pct	Abun
PROSNOT19	prostate, 59 M	3	0.0815	
PANCNOT04	pancreas, 5 M	4	0.0675	
LPARNOT02	parotid gland, 70 M	2	0.0649	
PANCNOT01	pancreas, 29 M	3	0.0642	
PROSNOT14	prostate, 60 M, match to PROSTUT08	2	0.0512	
PANCNOT07	pancreas, fetal M	1	0.0287	
PROSNOT18	prostate, 58 M	1	0.0256	
ISLTNOT01	pancreas, islet cells, M/F	2	0.0251	
PROSNOT15	prostate, 66 M. Match to PROSTUT10	1	0.0241	
BRSTNOT05	breast, 58 F, match to BRSTTUT03	1	0.0154	
PANCNOT05	pancreas, 2 M	1	0.0146	
PROSTUT04	prostate tumor, 57 M, match to PROSNOT06	1	0.0117	
LUNGAST01	lung, asthma, 17 M	1	0.0094	
PROSNON01	prostate, 28 M, NORM	1	0.0094	
CARDFEM01	heart, fetal, NORM, WM	1	0.0083	

FIGURE 5

## INTERNATIONAL SEARCH REPORT

Intern. Appl. No.

PCT/US 98/01440

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/57 C12N9/64 C12N1/21 C12P21/02 C07K16/40  
C12Q1/68

According to international Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	EP 0 297 913 A (AMGEN INC) 4 January 1989 see the whole document ---	1-11,17
X	WO 87 02709 A (BIOTECH RES PARTNERS LTD) 7 May 1987 see the whole document ---	1-11,17
X	ANGERMANN, A. ET AL.: "Cloning and expression of human salivary-gland kallikrein in Escherichia coli" BIOCHEM. J., vol. 262, 1989, pages 787-793, XP002065636 see the whole document ---	1-11,17

 Further documents are listed in the continuation of box C Patent family members are listed in annex

## \* Special categories of cited documents

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\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

PCT/US 98/01440

## C/Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of the relevant passages	Relevant to claim No
X	BAKER, A.R. AND SHINE, J.: "Human kidney kallikrein: cDNA cloning and sequence analysis" DNA, vol. 4, no. 6, 1985, pages 445-450, XP000106683 see the whole document ---	1-11,17
X	EVANS, B.A. ET AL.: "Structure and chromosomal localization of the human renal kallikrein gene" BIOCHEMISTRY, vol. 27, 1988, pages 3124-3129, XP002065637 see the whole document ---	1-11,17
X	FUKUSHIMA, D. ET AL.: "Nucleotide sequence of cloned cDNA for human pancreatic kallikrein" BIOCHEMISTRY, vol. 24, 1985, pages 8037-8043, XP002065638 see the whole document ---	1-11,17
X	WO 95 03334 A (MAYO FOUNDATION) 2 February 1995 see the whole document ---	11
A	WO 95 30758 A (MAYO FOUNDATION ;HYBRITECH INC (US); TINDALL DONALD J (US); YOUNG) 16 November 1995 see the whole document -----	1

## INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. Jinal Application No

PCT/US 98/01440

Patent document cited in search report	Publication date		Patent family member(s)	Publication date
EP 0297913	A 04-01-1989		AT 118547 T AU 2075588 A DE 3853023 D DE 3853023 T DK 91389 A ES 2068201 T FI 890889 A JP 1503679 T PT 87887 A,B WO 8900192 A	15-03-1995 30-01-1989 23-03-1995 08-06-1995 01-05-1989 16-04-1995 24-02-1989 14-12-1989 31-05-1989 12-01-1989
WO 8702709	A 07-05-1987		US 4861708 A AU 6549186 A DK 321987 A EP 0234095 A JP 63501900 T	29-08-1989 19-05-1987 24-06-1987 02-09-1987 28-07-1988
WO 9503334	A 02-02-1995		US 5516639 A AU 7252594 A CA 2165672 A EP 0710253 A JP 9501152 T ZA 9405364 A	14-05-1996 20-02-1995 02-02-1995 08-05-1996 04-02-1997 13-03-1995
WO 9530758	A 16-11-1995		AU 2639095 A CA 2189774 A EP 0804593 A JP 10500294 T ZA 9503727 A AU 5788996 A EP 0826056 A WO 9634964 A	29-11-1995 16-11-1995 05-11-1997 13-01-1998 07-08-1996 21-11-1996 04-03-1998 07-11-1996